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Entrainment Dissociates Transcription and Translation of a Circadian Clock Gene in *Neurospora*

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Summary

Circadian systems coordinate the daily sequence of events in cells, tissues, and organisms. In constant conditions, the biological clock oscillates with its endogenous period, whereas it is synchronized to the 24 hr light:dark cycle in nature. Here, we investigate light entrainment of *Neurospora crassa* to photoperiods that mimic seasonal changes. Clock gene (*frequency*, or *frq*) RNA levels directly reflect the light environment in all photoperiods, whereas the FRQ protein follows neither RNA levels nor light transitions. Induction of *frq* RNA and protein can be dissociated by as much as 6 hr, depending on photoperiod. The phase of entrainment at the physiological level (e.g., asexual spore development) correlates with FRQ protein. Thus, a dissociation of transcription, translation, and protein stability is fundamental to circadian entrainment of *Neurospora*. Our findings suggest that simple feedback models are insufficient to explain the molecular circadian mechanisms under entrained conditions and that clock control of light input pathways involves post-transcriptional regulation. The regulators mediating the dissociation between RNA and protein levels are still unknown and will be the key to understanding both circadian timing at the molecular level and how the clock exerts control over many cellular processes.

Results and Discussion

Although free-running rhythmicity is the most conspicuous quality of circadian clocks in laboratories, their most relevant feature is how they entrain to a 24 hr day in nature [1]. Only by understanding the molecular mechanisms and physiology of entrainment will we understand how the system works. The strongest entraining agent (zeitgeber; German for time-giver) is light. In most parts of the world, the daily amount of light varies greatly according to season. Organisms adapt by measuring changing day length (photoperiod) and using the circadian system to time seasonal events, such as reproduction in many plants and animals. The circadian clock also regulates the "phase" of entrainment (the time of day when a given regulated event occurs) and, in so doing, underlies the rich variation in human chronotypes, colloquially referred to as larks and owls [2].

Circadian oscillations are seen from the level of molecules to complex outputs such as behavior. A set of "clock genes" that form an autoregulatory, transcription/translation negative feedback loop has been described as the basis of circadian rhythmicity in all model organisms. Research into the molecular mechanisms of entrainment has primarily focused on how clock components are regulated after the introduction of light. The first demonstration therein showed a dramatic increase in clock gene RNA levels in *Neurospora* [3]. Subsequently, an alternative mechanism, namely light-dependent degradation of a clock protein (TIMELESS, in *Drosophila*), was described [4–6]. These mechanisms can explain phase shifts and have been recruited to describe entrainment [3, 7, 8]. Yet, how they mediate entrainment to full days and nights or how they contribute to the phenomenon of photoperiodism is far from understood.

The filamentous fungus, *Neurospora crassa*, is already well established as a simple model for the circadian system. The molecular clock mechanism is multioscillatory [9, 10] and receives light via multiple input pathways [11], as in plants and animals [12–14]. Most of *Neurospora*'s light responses depend on WHITE COLLAR-1 for light reception [15]. However, entrainment of *Neurospora*'s circadian physiology persists without *white collar-1* (*wc-1*) [11] but not without *frq* [9, 10, 16].

There is a wealth of information concerning regulation of *frq* RNA and FRQ protein in constant conditions. In constant light (LL), noisy but constitutively high *frq* RNA and protein levels are maintained [3, 17]. On transfer to constant darkness (DD), *frq* RNA first decreases and then starts to rise sometime between 8 and 12 hr, followed by FRQ protein some 4 hr later [18]; thereafter, *frq* and FRQ levels are controlled by the circadian clock, such that a wave of expression repeats about once per 22 hr [18, 19]. Thus, judged by the kinetics of *frq* RNA and protein in constant conditions (LL and DD), the clock is stalled during the light phase and appears to progress only at night. FRQ could, therefore, serve as an hourglass timer to measure night length. One can test this hypothesis relatively easily, simply by determining a molecular profile of *frq* and FRQ in a series of different entrainment conditions. We chose to systematically compare molecular light regulation in four different photoperiods, based on functional assays showing photoperiodism in *Neurospora* [20]. All light:dark (LD) cycles are 24 hr long, and they range from short days/long nights to long days/short nights.

In all photoperiods tested, *frq* RNA rapidly increases approximately 10-fold with the onset of light (Figure 1, left-most panel). Levels then drop (adapt) to less than 50% of peak values, where they remain for the rest of the light period. After the transition to darkness, *frq* rapidly decreases. In nights of 10 hr or longer (LD 4:20, 10:14, and 14:10), *frq* levels start to rise approximately 8 hr after lights-off, independent of photoperiod (for a $p < 0.01$, the increase in 4:20 and 14:10 LD cycles occurs

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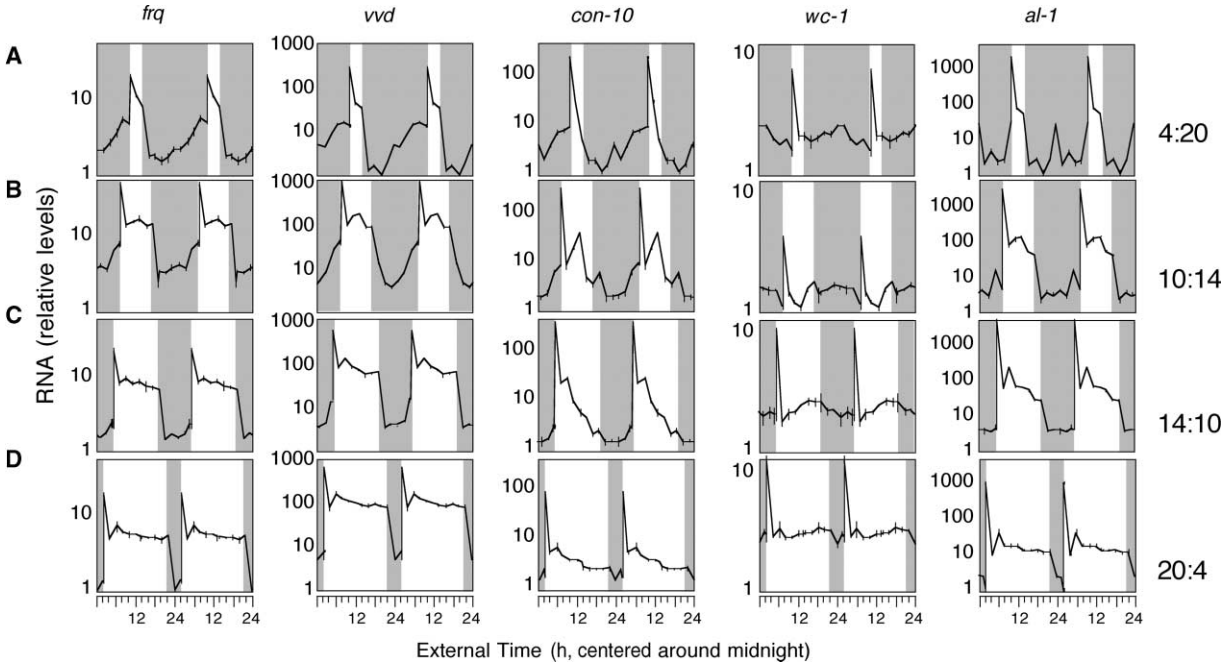

Figure 1. Light-Regulated RNA Expression and Photoperiod

Tissue was grown for 5 days in photoperiodic conditions (LD cycles of 4:20 [A], 10:14 [B], 14:10 [C], and 20:4 [D], indicated on the left). The gray areas correspond to the dark portions of the cycles. Samples were harvested every 2 hr throughout each cycle, in addition to a sample collected 15 min after lights-on. RNA levels, determined by RT-PCR (see Supplemental Data), were normalized to those of ribosomal RNA (not shown) and, in addition, between the different photoperiods. Each original data set over 24 hr is drawn twice in sequence. Each point is the average of three replicates of a single sample, with error bars representing standard deviations. Time courses for *frq*, *vvd*, *con-10*, *wc-1*, and *al-1* RNAs are graphed from left to right, respectively. Here, and throughout the paper, external time is used [44] to indicate the coordinates of the LD cycle. External Time 0 always corresponds to the middle of the dark period. Note that all RNA values are plotted on a logarithmic scale.

at 8 hr, and in 10:14 at 10 hr). At least at the RNA level, *frq* expression appears to be driven by light, as can be seen by the acute responses to lights-on and -off and the delayed response to lights-off. As such, *frq* RNA behaves like an hourglass timer.

Is the driven response of *frq* RNA specific for this gene, or is this a general feature of light-dependent transcription in *Neurospora*? In addition to *frq*, many other light-regulated RNAs have been described. Some are coregulated by light and the circadian clock (*vvd* [21] and *con-10* [22]), whereas others are only light responsive and show no circadian rhythmicity in DD (*wc-1* [23, 24] and *al-1* [24]). We investigated the RNA expression profiles of these four genes from the same tissues grown in different photoperiods. *vvd* RNA encodes a protein that contributes to photo-adaptation in *Neurospora* [21, 25, 26] and thus could regulate light signaling pathways that contribute to entrainment. In all photoperiods, the expression profiles of *vvd* RNA are similar to those of *frq*, except for their higher amplitude (Figure 1, second panel from left). *con-10* is a gene involved in conidiation. Its response strength lies between that of *frq* and *vvd* (Figure 1, center panel), but unlike these, its RNA levels adapt down to dark levels in long days. As in the case of *frq* RNA, *vvd* and *con-10* spontaneously increase in longer nights, with a constant delay of approximately 8 hr after lights-off.

Among the light-induced RNAs that are not controlled by the circadian clock in DD, *wc-1* encodes a blue light receptor that also acts as a transcription factor to directly regulate *frq* expression [27, 28]. In all photoperiods, *wc-1* RNA exclusively shows the acute response to lights-on (Figure 1, second panel from the right); it immediately returns to baseline levels, where it remains until the next experimental dawn. The adaptation kinetics of *al-1* RNA (Figure 1, rightmost panel) are similar to those of *frq* and *vvd*. The fact that neither of the noncircadian genes, *wc-1* nor *al-1*, are upregulated during prolonged exposure to darkness indicates that the spontaneous increase of *frq*, *vvd*, and *con-10* is related to the mechanisms that ensure rhythmicity of these genes (for at least one cycle) in DD.

Thus, all five RNA profiles mirror the light environment: a rapid response to lights-on (resembling a "shock" response), adaptation, and (depending on their level at experimental dusk), a rapid decrease in response to lights-off. Even the spontaneous increase in *frq*, *vvd*, and *con-10* appears to be triggered by lights-off, with a constant delay of 8 hr (see Table S2). This is consistent with earlier results [10] from an investigation of entrainment of *Neurospora* at the physiological level in LD cycles of different total length (e.g., 16 or 20 hr) but always with 50% light and 50% darkness. In these symmetrical LD cycles, *Neurospora* forms the daily bands of asexual spores approximately 7 hr after lights-off, independent of cycle length. This indicates a light-driven system, which is inconsistent with circadian clocks in most other organisms, where entrainment is characterized by differ-

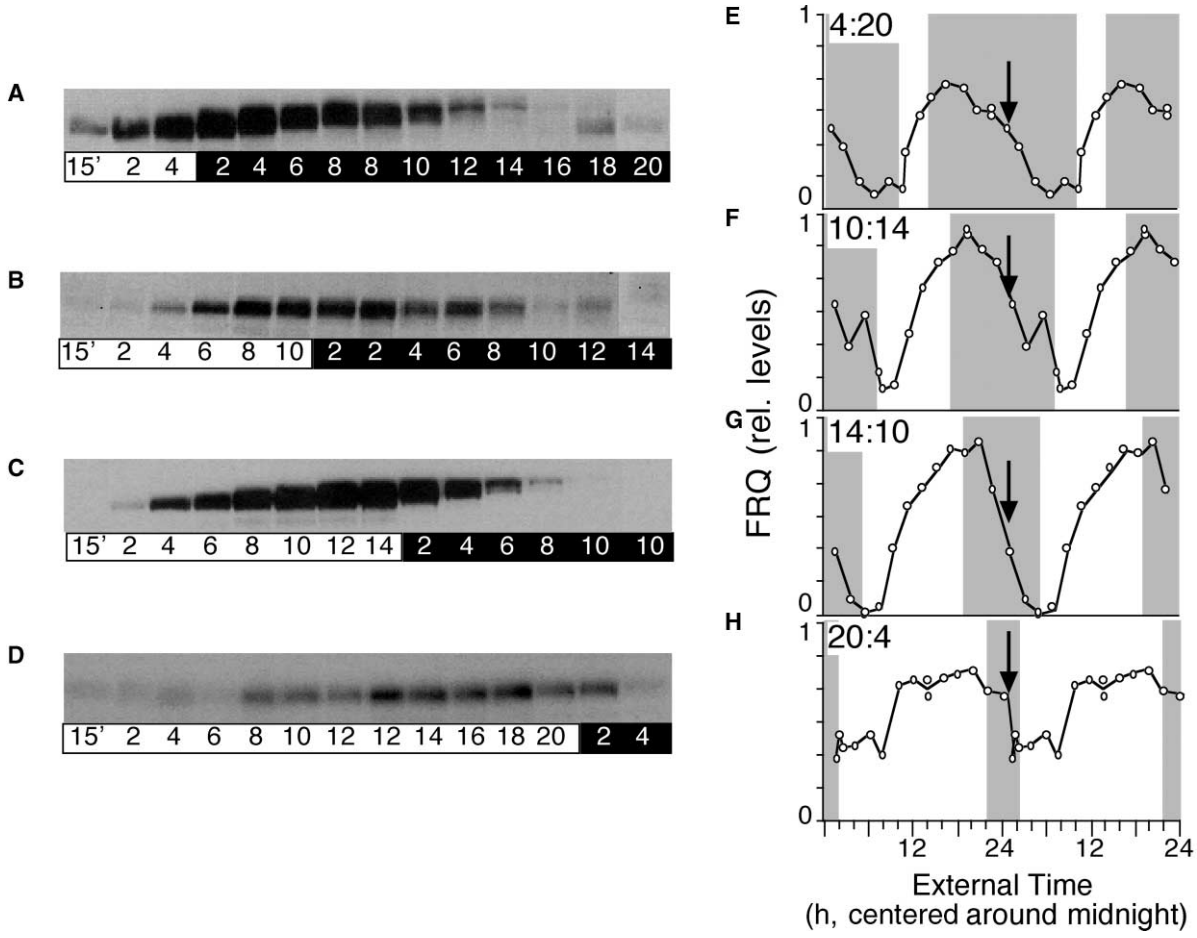

Figure 2. Clock Protein Expression and Photoperiod

FRQ protein expression determined from the same tissues used in Figure 1 is shown as Western blots ([A–D], LD cycles as in Figure 1 [A–D]) and their quantification (E–H). The dark portions of the different LD cycles are indicated by black bars (A–D) or gray areas (E–H). Developmental controls that covered the beginning and the end of the experiment are shown in each series as duplicate time points (e.g., two 8 hr time points in the 4:20 LD cycle). Quantifications [11] are double-plotted as in Figure 1; arrows indicate half-maximal levels of FRQ, with respect to its decrease.

ent but systematic phase relationships to the zeitgeber depending on cycle length. *Neurospora* does, however, establish systematic phase angles in different zeitgeber cycles, both on the RNA and physiological levels, when temperature is used for entrainment rather than light [10].

Because *frq* has been implicated in controlling phase shifts in many different experiments [3, 19], we investigated the FRQ protein profiles in the different photoperiodic cycles (Figure 2). Several features are similar to profiles obtained from tissue grown in DD, namely, protein concentrations oscillate over a circa-24 hr period, and the mobility of the protein decreases prior to its disappearance (due to increasing phosphorylation [18]; Figures 2A–2D). In contrast to DD, where the timing (or phase) of the FRQ oscillation is strictly determined by the last transfer from light, the phase of the FRQ oscillation in LD cycles is not keyed to these transitions. After long nights (e.g., in LD 4:20, Figures 2A and 2E), the protein surges up to high levels within 2 hr of lights-on, as in experiments in which tissue is grown in DD and transferred to LL [17]. However, the response to lights-on after shorter nights is in contrast to all prior observations. A significant increase in FRQ levels can be delayed by as much as 8 hr into the light phase (e.g., in LD 20:4, Figures 2D and 2H). In long nights, the surge of FRQ coincides with lights-on, whereas it is progressively delayed in shorter nights/longer days. Depending on photoperiod, FRQ protein declines with different rates (slopes of linear regressions through the nocturnal declines: LD 4:20, −0.05; LD 10:14, −0.07; LD 14:10, −0.12; LD 20:4, −0.21), so that it reaches approximately half-maximal levels around 1 hr past midnight in night lengths from 4 to 20 hr (arrows in Figure 2). In the case of the LD 20:4, the RNA and protein actually decline coincidentally and obscure the role of negative feedback on *frq* RNA levels. In the context of the entrainment protocols used here, FRQ levels are independent of dawn or dusk.

Yet, so far all physiological results (i.e., entraining asexual spore formation in light cycles of different lengths and in 24 hr photoperiod cycles) showed a driven response rather than circadian entrainment [10, 16]. We, therefore, revisited entrainment in varying photoperiods

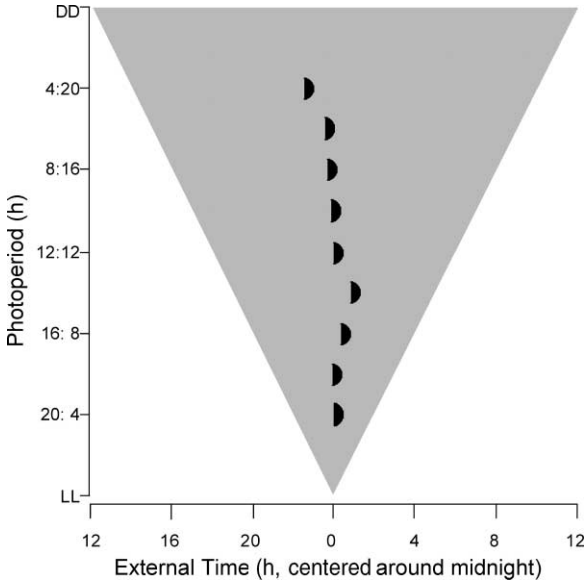

Figure 3. Regulation of Asexual Spore Formation in Different Photoperiods

The phase of the onset of spore formation (the time of 50% of maximum value) was determined for the clock wild-type strain (*band*) with the race tube assay [45]. Analysis was performed with the Chrono program [46].

within a 24 hr day (Figure 3). The results are strikingly different to prior observations. In all photoperiods between 4 and 20 hr, the onset of asexual spore formation (conidiation) is around midnight (Figure 3A). Thus, the phasing of conidiation is, like FRQ protein and unlike *frq* RNA, independent of light transitions. Although FRQ, even as a constitutively expressed component, is essential for light-regulated conidiation [24], the biochemical relationship between FRQ and the conidiation pathway has not been elucidated. These data suggest that high FRQ levels suppress conidiation whereas FRQ decline supports it.

FRQ is a critical clock component in *Neurospora* and is required for circadian entrainment with light [9, 10, 16]. Previous experiments in DD established that FRQ negatively feeds back onto its own expression [19], allowing *frq* transcription only when the concentration of its protein falls below a threshold of approximately 10 copies per nucleus [29]. The negative, autoregulatory feedback involving *frq* and FRQ is important for sustaining the oscillation in DD, but *frq* fails to oscillate in light cycles and thus is not imperative for maintaining a running clock under entrained conditions. This conclusion is not completely novel, in that it was previously shown that the accumulation of *timeless* RNA and protein (key components of the *Drosophila* clock feedback loop) are superimposed in cycles that are shorter than the free running period [30].

Temporal programs such as the circadian clock integrate environmental history and use this "memory" to endogenously generate functional niches within the daily or seasonal cycle. Our results show that expression of a *Neurospora* clock component, FRQ protein, is history dependent. This contrasts with *frq* RNA expression, which appears to be driven by light. Because *frq* transcription is acutely induced by light regardless of FRQ concentration, it was hypothesized that light overrides the negative feedback by FRQ [3]. The entrainment results shown here indicate a more complex regulation, beyond the transcriptional feedback loop, involving posttranscriptional regulation of the *Neurospora* circadian clock. Depending on the structure of the light:dark cycle, for example, the declines of *frq* RNA and protein in darkness either occur with a lag or coincide. Candidates for the components responsible for this posttranscriptional regulation have been detected in microarray experiments (the expression of translational and posttranslational regulators show circadian oscillation), and genetics and biochemistry already suggest determinants of clock protein production and destruction [31–36]. The photoperiod-specific turnover kinetics of FRQ and regulation of conidial band formation also show that the circadian clock in *Neurospora* must be running in LD cycles and not only during darkness, as has been suggested by most prior observations [7]. Without this feature, one of the central qualities of a circadian system, namely the ability to systematically vary the phase angle of entrainment depending on zeitgeber conditions, i.e., in different seasons [37], would be lost.

Given that *Neurospora* is considered a model system for higher eukaryotes, will similar mechanisms be described in mammals? In hamsters and mice, the expression of selected clock gene RNAs and proteins in the clock pacemaker in the brain (the suprachiasmatic nucleus, SCN) generally mirrors the light cycle in long and short days [38, 39]. However, as shown for the mammalian clock gene, *Per1*, entrainment of gene expression can be dissociated from entrainment of SCN neurophysiology and behavior [40]. This is comparable to dissociation of clock gene RNA versus protein levels and behavior in *Neurospora*.

Furthermore, the circadian system modulates numerous sensory transduction pathways, so that responses to identical stimuli vary according to the time of day. The dissociation between an acute RNA and a delayed protein response, as shown here, could be one of the mechanisms by which the clock mediates its control over transduction pathways. Such a regulation would not be detected in DNA microarray experiments. Similar types of regulation could also be present in the clock control of complex outputs as diverse as photosynthesis [41], hormone release [42], or circadianly gated cell division [43].

Supplemental Data

Supplemental Experimental Procedures are available with this article online at http://www.current-biology.com/cgi/content/full/14/5/433/DC1/.

Acknowledgments

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